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Effects of Proteolytic Enzyme Inhibitors as Absorption Enhancers on the Transdermal Iontophoretic Delivery of Calcitonin in Rats

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Abstract—The effects of proteolytic enzyme inhibitors, aprotinin, soybean trypsin inhibitor and camostat mesilate as absorption enhancers on the transdermal iontophoretic delivery of salmon calcitonin (SCT) have been examined in rats. The dermal absorption of SCT was evaluated with hypocalcaemic effect. Application of SCT (12·5 int. units/rat) onto abdominal skin did not produce any hypocalcaemic effect. This produced a small hypocalcaemic effect with cationic iontophoresis (drug phase, anode; reference phase, cathode; high frequency pulses of 1 V at 10 kHz, 2h). Furthermore, camostat mesilate (1 mM) and aprotinin (10^6 int. units mL^{-1}) enhanced the hypocalcaemic effects on the application of SCT with iontophoresis. These hypocalcaemic effects were highest with the pH 4·0 preparation compared with those of the pH 5·5, pH 7·0 and pH 8·0 preparations. However, soybean trypsin inhibitor did not change the hypocalcaemic effects. This was because the soybean trypsin inhibitor is a relatively high molecular weight peptide (mol. wt 8000) and an anion at used pH, and therefore was not absorbed through rat skins with cation iontophoresis.

The systemic delivery of polypeptides through the parenteral route has recently received much attention. Since polypeptides are extensively degraded by proteolytic enzymes in the gastrointestinal tract, their bioavailability is extremely poor when taken orally. The transdermal delivery of polypeptides can be considered as an available route (Meyer et al 1988). However, the stratum corneum of skin is a high transport barrier against percutaneous absorption of most substances, including polypeptides and proteins. Approaches for transdermal delivery have included prodrug, permeating enhancer and iontophoresis. The use of the iontophoresis technique to facilitate the transdermal delivery of uncharged or charged molecules, including macromolecules has recently been the subject of numerous studies (Tyle 1986; Burnette & Marrero 1986). Besides the transport barrier, the enzymatic barrier, produced by peptidase is important in limiting the transdermal delivery of polypeptides (Zhou & Po 1990).

We previously reported the effect of proteolytic enzyme inhibitor as absorption enhancer on transdermal iontophoretic delivery of vasopressin (mol. wt 1084) and its analogue in rats (Morimoto et al 1992). This result showed that camostat mesilate enhanced the dermal absorption of vasopressin and its analogue. In the present study, the effects of proteolytic enzyme-inhibitors, aprotinin and soybean trypsin inhibitor and camostat mesilate as an absorption enhancer on the transdermal iontophoretic delivery of salmon calcitonin (SCT) were examined in rats. Calcitonin (mol. wt 3500) which is a cyclic 32 amino acid peptide has been used in the treatment of Paget's disease, osteoporosis and hypercalcaemia (Stevenson & Evans 1981).

Materials and Methods

Materials

Synthesized salmon calcitonin (SCT) was supplied from Teikokuzoki (Tokyo). Aprotinin and soybean trypsin inhibitor were purchased from Sigma Chemical Inc. (St Louis, MO). Camostat mesilate (FOY-305) was supplied from Ono Pharm. Co. Ltd (Osaka). All other chemicals were of reagent grade.

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Preparations

SCT was dissolved in the buffer solutions (0·2 M disodium phosphate buffer: 0·1 M citric acid buffer; pH 4·0, 5·5, 7·0 and 8·0). Proteolytic enzyme-inhibitors, aprotinin (10^3 int. units mL^{-1}), soybean trypsin inhibitor (6·25 mM) and camostat mesilate (1 mM) were dissolved in drug solution. The final pH of preparations was adjusted by adding HCl or NaOH solution.

Transdermal iontophoretic systems

The percutaneous absorption of SCT was evaluated by its hypocalcaemic effect. Male Wistar rats (230–250 g), from which the abdominal hair area was removed with electric hair clippers and an electric razor, were fasted for 20 h before the experiments, but water was given freely. During the experiment, the rats were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}).

The iontophoretic experiments were carried out by a method described in our previous paper (Fig. 1) (Morimoto et al 1992). Two polyethylene cylinder cells (20 mm i.d. × 15 mm; available skin area 3·14 cm^2) were fixed onto the rat abdominal skin surface by an adhesive agent, Alon Alpha (Sankyo Co. Ltd, Tokyo) at an interval of 1 cm. One cell (drug phase) was filled with 2 mL of drug solution and another cell (reference phase) was filled with 2 mL of 0·9% w/v NaCl solution (saline) after obtaining constant urine volume. A pair of Ag/AgCl electrodes was immersed in the solutions with anode in the drug solution and cathode in saline. These electrodes were connected to the transdermal iontophoretic system, a power source (high frequency pulse of 1 V at 10 kHz).

Blood samples (0·2 mL) were obtained with a heparinized syringe from the femoral vein 5 min before and at 30, 60, 120

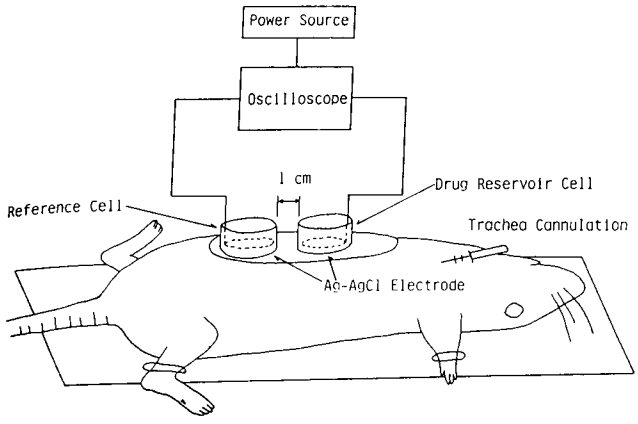

FIG. 1. Iontophoresis technique in the rat.

and 150 min post-dose. The plasma was separated by centrifugation at 3000 rev min^{-1}. The plasma calcium levels were determined by Calcium C Test Wako (Wako Pure Chemicals Ind., Osaka).

Data analysis

The area above the hypocalcemic effects curve (AAC) was calculated by means of trapezoidal integration by using the program MULTI (Yamaoka et al 1981). Statistical significance of data was assessed by using Student's paired *t*-test.

Results

Application of SCT (12·5 int. units/rat) onto abdominal skins did not produce any hypocalcemic effect in rats. Application of SCT (pH 4·0) by cationic iontophoresis (high frequency pulses of 1 V at 10 kHz, 2 h) produced small hypocalcaemic effects (Fig. 2). However, the application of SCT (pH 5·5, pH 7·0 and pH 8·0) by iontophoresis did not produce the hypocalcaemic effects. This may be because SCT, with an isoelectric point of 6·5, is a cation at pH 4·0 and an anion at pH 8·0. When the iontophoresis current was reversed by placing the cathode in the drug phase (pH 8·0) and anode in the reference phase, hypocalcaemic effects were not observed.

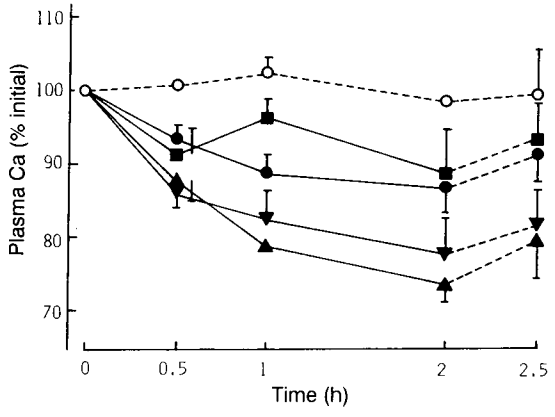

FIG. 2. Effects of proteolytic enzyme inhibitors on the changes in plasma calcium levels induced by iontophoresis (high frequency pulses of 1·0 V at 10 kHz, 2 h) following application of SCT (12·5 int. units/rat, pH 4·0) onto abdominal skin in rats. ○ Without proteolytic enzyme inhibitors and iontophoresis, ● without proteolytic enzyme inhibitors, ▲ camostat mesilate (1 mM), ▼ aprotinin (10^3 int. units mL^{-1}), ■ soybean trypsin inhibitor (6·25 mM). Each point represents the mean ± s.e.m. of 4 animals.

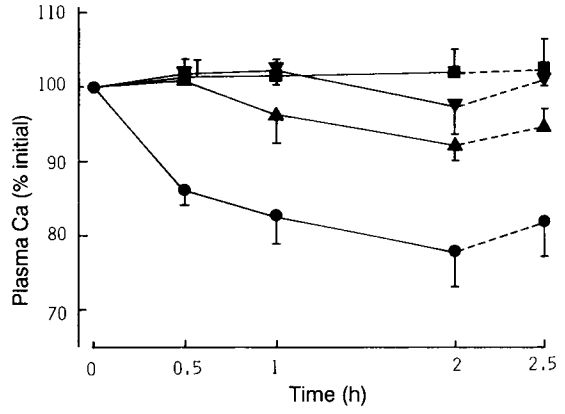

FIG. 3. Effects of pH of preparations on the changes in plasma calcium levels induced by iontophoresis (high frequency pulses of 1·0 V at 10 kHz, 2 h) following application of SCT (12·5 int. units/rat) preparation containing aprotinin (10^3 int. units mL^{-1}) onto abdominal skin in rats. pH of preparations; ● pH 4·0, ■ pH 5·5, ▲ pH 7·0, ▼ pH 8·0. Each point represents the mean ± s.e.m. of 4 animals.

Fig. 2 shows the effects of proteolytic enzyme-inhibitors, aprotinin and soybean trypsin inhibitor, and camostat mesilate on hypocalcaemic effects after transdermal administration of SCT (12·5 int. units/rat) preparations (pH 4·0) by cationic iontophoresis in rats. Aprotinin (10^3 int. units mL^{-1}) and camostat mesilate (1 mM) enhanced the hypocalcaemic effects on the administration of SCT. However, soybean trypsin inhibitor (6·25 mM) did not enhance the hypocalcaemic effect on the administration of SCT.

Fig. 3 shows the effects of pH (pH 4·0, 5·5, 7·0 and 8·0) of preparations on the hypocalcaemic effects after transdermal administration of SCT (12·5 int. units/rat) preparations containing aprotinin (10^3 int units mL^{-1}) by cationic iontophoresis in rats. The pH 4·0 preparation showed the highest hypocalcaemic effects compared with those preparations of pH 5·5, 7·0 and 8·0.

Fig. 4 shows the effects of pH (pH 4·0, 5·5, 7·0 and 8·0) of preparations, on the hypocalcaemic effects after transdermal

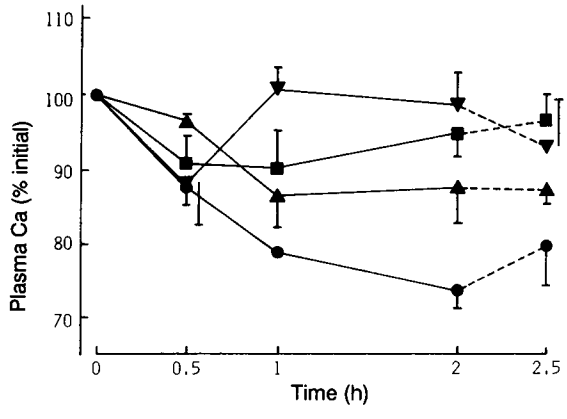

FIG. 4. Effects of pH of preparations on the changes in plasma calcium levels induced by iontophoresis (high frequency pulses of 1·0 V at 10 kHz, 2h) following application of SCT (12·5 int. units/rat) preparation containing camostat mesilate (1 mM) onto abdominal skin in rats. pH of preparations; ● pH 4·0, ■ pH 5·5, ▲ pH 7·0, ▼ pH 8·0. Each point represents the mean ± s.e.m. of 4 animals.

Table 1. Area above the hypocalcaemic effect response curve ($AAC_{0\text{-}2.5\ h}$) after application of SCT preparations containing proteolytic enzyme inhibitors with iontophoresis (high frequency pulses of 1·0 V at 10 kHz, 2 h) onto rat abdominal skins.

	$AAC_{0\text{-}2.5\ h}$ (% Ca h)
SCT (12·5 int. units/rat)	
Without proteolytic enzyme inhibitors	23·87 ± 4·244
Camostat mesilate (1 mM)	46·77 ± 2·110[a]
Aprotinin (10^3 int. units mL^{-1})	41·25 ± 5·309[b]
Soybean trypsin inhibitor (6·25 mM)	20·01 ± 9·446

The AAC was calculated using the trapezoidal method. [a] Significantly different from without camostat mesilate at $P < 0.005$. [b] Significantly different from without camostat mesilate at $P < 0.05$. Each value represents the mean ± s.e.m. of 4 animals.

administration of SCT (12·5 int. units/rat) preparations containing camostat mesilate (1 mM) with cationic iontophoresis in rats. The preparation at pH 4·0 showed the highest hypocalcaemic effect compared with preparations at pH 5·5, 7·0 and 8·0.

Soybean trypsin inhibitor (6·25 mM) did not affect the hypocalcaemic effects after transdermal administration of SCT preparations at pH 5·5, 7·0 and 8·0 with the cationic iontophoresis.

The area above the hypocalcaemic effect curve (AAC) in rats after application of SCT preparations containing camostat mesilate and aprotinin by cationic iontophoresis are summarized in Table 1. $AAC_{0\text{-}2.5\ h}$ of SCT preparations containing camostat mesilate (1 mM) and aprotinin (10^3 int. units mL^{-1}) increased 2·0 and 1·7 times, respectively, compared with those without proteolytic enzyme inhibitor.

Discussion

Polypeptides, which are hydrophilic and macromolecular compounds, did not permeate through the normal skin. The stratum corneum of epidermis is the main barrier limiting the passive transdermal diffusion of these compounds. Application of iontophoresis enhanced the skin permeation of peptides such as insulin (mol. wt 6500), leuprolide (mol. wt 1200) and thyrotropin releasing hormone (TRH; mol. wt 365) (Burnette & Marrero 1986). In this study, SCT was not absorbed through abdominal skin in rats. Absorption of SCT, which was a cation at pH 4·0, was slightly enhanced by application of cationic iontophoresis, however, absorption of SCT, which was an anion at pH 8·0, was not enhanced by anion iontophoresis. This was because at pH greater than 4, skin carries a negative charge and acts as a cation-selective membrane (Burnette & Marrero 1986).

The peptidase activities in dermal tissue of rats were in the following order; aminopeptidase > cathepsin-B > trypsin (Morimoto et al 1992). Zhou & Po (1990) showed that leucine aminopeptidase activity levels in dermal, nasal, buccal and rectal tissues were the same when adjusted for protein content. Therefore, proteolytic enzymatic degradation would function as one barrier, among others, for transdermal delivery of peptide. Camostat mesilate inhibited the activities of aminopeptidase and trypsin in dermal tissue of rats, while aprotinin and soybean trypsin inhibitor inhibited only trypsin activity in dermal tissue of rats. Camostat mesilate enhanced the transdermal absorption of vasopressin and its analogue with cationic iontophoresis. Whereas, aprotinin and soybean trypsin inhibitor did not enhance the transdermal absorption of vasopressin and its analogue (Morimoto et al 1992). In this study, camostat mesilate (1 mM) and aprotinin (10^3 int. units mL^{-1}) enhanced the transdermal absorption of SCT with cationic iontophoresis in rats. However, soybean trypsin inhibitor did not change the transdermal absorption of SCT with the iontophoresis in rats. This result of aprotonin did not agree with our previous result of vasopressin (Morimoto et al 1992). Parsons et al (1979) reported that the plasma levels of SCT injected subcutaneously were enhanced with aprotinin. The Cys-1-Cys-7 ring of SCT, a 32-residue peptide is in close association with the helix between residues 8 and 22, while the C-terminal decapeptide folds back toward the core, forming a loose loop (Meyer et al 1991). The C-terminal of the decapeptide is known to be important for biological activities.

The transdermal absorption of the proteolytic enzyme inhibitor is one of the important factors in the absorption enhancing effect of the proteolytic enzyme inhibitor. Camostat mesilate, which is a relatively low molecular weight compound and a cation in the range of pH used, was slightly absorbed through rat abdominal skin and this absorption was enhanced with iontophoresis (Morimoto et al 1992). Absorption of aprotinin (mol. wt 6500), which is a peptide (pI = 10·5) and cation in the range of pH used, might be enhanced through rat abdominal skin with cationic iontophoresis. However, soybean trypsin inhibitor (mol. wt 8000), which is a peptide (pI = 4·0–4·2), and anion or neutral in the range of pH used, might not permeate into skin with cationic iontophoresis.

In conclusion, the co-application of a proteolytic enzyme inhibitor such as camostat mesilate and aprotinin, and iontophoresis will be useful for transdermal delivery systems of SCT.

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